Environmental Toxicology and Chemistry—Volume 41, Number 10—pp. 2342–2352, 2022
Received: 12 February 2022 | Revised: 2 April 2022 | Accepted: 14 July 2022

Critical Perspectives

Digital Video Acquisition and Optimization Techniques for Effective Animal Tracking in Behavioral Ecotoxicology

Jason Henry, Yutao Bai, and Donald Wlodkowic*

The Neurotox Lab, School of Science, RMIT University, Melbourne, Victoria, Australia

Abstract: Behavioral phenotypic analysis is an emerging and increasingly important toolbox in aquatic ecotoxicology. In this regard digital video recording has recently become a standard in obtaining behavioral data. Subsequent analysis requires applications of specialized software for detecting and reconstructing animal locomotory trajectories as well as extracting quantitative biometric endpoints associated with specific behavioral traits. Despite some profound advantages for behavioral ecotoxicology, there is a notable lack of standardization of procedures and guidelines that would aid in consistently acquiring high-quality digital videos. The latter are fundamental for using animal tracking software successfully and to avoid issues such as identification switching, incorrect interpolation, and low tracking visibility. Achieving an optimized tracking not only saves user time and effort to analyze the results but also provides high-fidelity data with minimal artifacts. In the present study we, for the first time, provide an easily accessible guide on how to set up and optimize digital video acquisition while minimizing pitfalls in obtaining the highest-quality data for subsequent animal tracking. We also discuss straightforward digital video postprocessing techniques that can be employed to further enhance tracking consistency or improve the videos that were acquired in otherwise suboptimal settings. The present study provides an essential guidebook for any aquatic ecotoxicology studies that utilize digital video acquisition systems for evaluation of behavioral endpoints. *Environ Toxicol Chem* 2022;41:2342–2352. © 2022 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Animal; Behavior; Tracking; Video; Computer; Ecotoxicology

INTRODUCTION

Pharmaceuticals, pesticides, personal care products, industrial additives, and surfactants are increasingly emitted from manufacturing, mining, and wastewater-treatment plants into aquatic ecosystems (Gaw et al., 2014; Grandjean & Landrigan, 2006). The increase in exposure to these pollutants poses long-term and poorly explored risks on diverse aquatic species at environmentally relevant concentration levels that can potentially lead to a decline of long-term ecological fitness (Grandjean & Landrigan, 2006; Zala & Penn, 2004). The data on environmental neurotoxicity provided by conventional bioassays are very limited, and the adverse impacts on the central nervous system of aquatic species at environmentally relevant concentrations remain largely unexplored (Grandjean & Landrigan, 2006; Zala & Penn, 2004).

The analysis of neuromodulatory and/or neurotoxic effects on exposure to ecologically relevant levels of contaminants is often based on analysis of animal behavior (Legradi et al., 2018). Behavior represents a highly integrative physiological endpoint that encompasses the context of fully functional central and peripheral nervous systems and all other organ systems. It thus is increasingly regarded as a dynamic and very sensitive sublethal parameter that can provide early indication of a chemical risk (Ågerstrand et al., 2020; Bownik & Wlodkowic, 2021; Henry & Wlodkowic, 2019; Melvin & Wilson, 2013). As a result, behavioral endpoints are being progressively adopted in aquatic ecotoxicology research (Bai et al., 2021; Brooks, 2009; Ford et al., 2021; Kalueff et al., 2016).

To date, many behavioral experiments are still performed at very small sampling scales and often scored manually. This is a tedious, time-consuming process, prone to analytical bias and low reproducibility. It is also prohibitive for any practical applications of higher-throughput risk-assessment paradigms, for

This article includes online-only Supporting Information.

* Address correspondence to donald.wlodkowic@rmit.edu.au
Published online 18 July 2022 in Wiley Online Library (wileyonlinelibrary.com).
DOI: 10.1002/etc.5434

instance, in prioritization screening of new production chemicals. Over the last decade, digital video file acquisition coupled with subsequent computer-based animal tracking has been increasingly adopted as the gold standard to obtain behavioral data sets (Ford et al., 2021). Digital animal tracking alleviates many bottlenecks associated with manual scoring while providing unbiased quantitative data sets.

Despite some profound advantages for behavioral ecotoxicology, there is a notable lack of standardization of procedures and guidelines that would aid in consistently acquiring high-quality digital videos (Ford et al., 2021; Henry & Wlodkowic, 2020). We postulate that the actual importance of video quality in obtaining high-fidelity and reproducible behavioral data sets is still greatly underappreciated by many in the field. In the present study, we draw attention to this important topic and illustrate key concepts in video-based behavioral analysis. We also provide recommendations on how to optimize video acquisition during the experimental setup to improve the analytical efficiency, consistency, and fidelity of behavioral data sets.

DIGITAL VIDEO DATA ACQUISITION

To obtain biometric behavioral data, digital video files have to be initially recorded. This is typically realized through placement of a camera vertically or horizontally above or in front of a test chamber (e.g., a microtiter plate, Petri dish, or fish tank) that cages the animal while providing it enough "space to behave" (Kohler et al., 2018). The activity of the animals is then recorded on the camera sensor in the form of pixels and saved as digital video files (Figure 1). Below we outline some concepts that are critical for successful implementation of digital video recording.

Sensor resolution

It is important to understand that every digital video file is characterized by a specific resolution. The latter depends on the number of pixels per area of the camera sensor. Most modern digital cameras can record in high-definition (HD; 1920 × 1080 pixels) or even ultrahigh-definition (UHD; 3840 × 2160 pixels) resolution (Figure 1). The higher the resolution of the camera sensor, the higher the effective pixel quantity that is available per organism. The majority of tracking systems for small model organisms recommend a minimum value of pixels (px) to be allocated to each individual organism for accuracy of detection and identity preservation during tracking (Henry & Wlodkowic, 2020; Rodriguez et al., 2018). In applied animal tracking software, objects are very difficult to

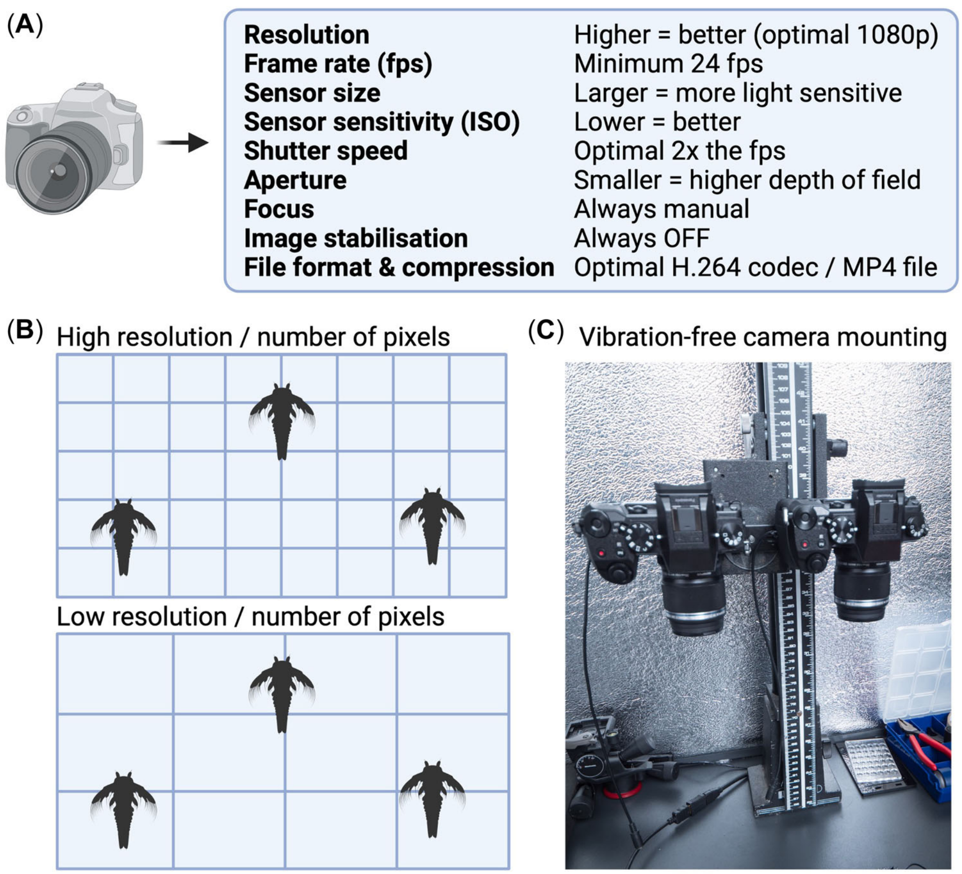

FIGURE 1: Digital video recording and its considerations for behavioral experiments. (**A**) Camera settings imperative to acquire high-quality and consistent video data. (**B**) Resolution of the camera sensor translates directly to the accuracy of subsequent animal tracking. (**C**) Stable, vibration-free, and consistent mounting of the camera and test chambers is critical for repeatable behavioral experiments. fps = frames per second; ISO = International Organization for Standardization.

effectively distinguish from the background of the video below such a pixel threshold. In fact, the majority of animal tracking software commonly employed in aquatic ecotoxicology such as commercial (Ethovision XT, LolliTrack, Zantix) and open source (ToxTrack) do require an approximately 50-px detection threshold for the object to be detected on a standard video frame (assumed to be 720 or 1080 px).

Thus, it follows that increasing the resolution of the camera increases the number of organisms that can be accurately tracked in an experiment. This is of high importance in experiments with small organisms commonly used in ecotoxicity testing such as rotifers as well as neonates of *Daphnia magna* and *Artemia franciscana* (Henry et al., 2019).

Sensor light sensitivity

The size of individual pixels on the sensor determines how much light it can detect. The larger the pixels and in translation the overall sensor size, the more light it can receive (Figure 1). As a result, large full-frame digital sensors can record higher-quality images in low-light conditions. However, it must also be remembered that cameras with large sensors feature smaller effective depth of field with lenses of identical focal length. As a result, such cameras might require stepping down the aperture setting on the objective lenses to be able to provide sharp images. This will in turn will reduce the amount of light falling on the sensor.

The sensitivity of a camera sensor to light can be altered through its electronic signal gain (International Organization for Standardization [ISO]) settings. Increasing the ISO will brighten the captured image. However, increasing the ISO setting will also increase the digital noise. The latter is often referred to as amount of "grain" in the video footage. The graininess of the video induces dynamic pixel intensity changes that can align with both the background and the target organism, resulting in a plethora of identity preservation issues in detection. For minimizing digital noise coming out of the camera sensors, we recommend using the standard manufacturers' base ISO setting and altering external lighting sources if additional light is required to adequately illuminate the samples, provide proper exposure, and detect the target organisms. The camera sensors when operating at base or close to base ISO settings (i.e., without excessive gain amplification of the incoming light signal) provide minimum digital noise and very clean video files. This in turn minimizes time-consuming digital processing of video files to remove the digital noise. In addition, it is important to note that increasing a white light source intensity too high can induce behavioral changes in some organisms. The zebrafish larval photomotor response assay is an example of altering white light intensity to induce a startle response (Henry et al., 2022; Walpitagama et al., 2019).

Infrared conversions of sensors

Some cameras can be altered to record video footage in infrared light (IR; >700 nm). On most digital cameras this typically involves the removal of the low-pass filter located on the sensor (largely dependent on the camera model) and the addition of an IR filter on the front element of the lens. The removal of the low-pass filter enables the camera to record the full spectrum of visible light. The addition of an IR high-pass filter on the front of the lens ensures that only IR light will be seen by the camera sensor. This setup also requires IR illumination sources to be employed in all experiments. We recommend using filters and corresponding IR illumination sources with the wavelength of light of approximately 850 nm. These wavelengths are advantageous because they allow for illumination of organisms in complete darkness. Furthermore, most animals are incapable of seeing this spectrum of light, enabling continuous IR illumination to be combined with a variety with photic sensory-motor assays (Paskin et al., 2014). In particular, diverse photic stimuli that utilize, for example, strong white light can be used because none of those stimuli will be seen by the camera. Without such IR conversion, any strong light stimuli would significantly alter the preset camera exposure.

It is important to note that on some current cameras the low-pass filter is combined with the antialiasing filter and that the removal of these filters will change the calibration of the camera, effectively disabling any autofocus function.

Video frame rate

The sensors of modern cameras enable recording of video files created from many still frames acquired in a unit of time. The video files are thus always characterized by the specific frame rate at which they are recorded. This parameter denotes the number of still frames acquired per second. Hence, it is often referred to in the camera settings as the frames per second (fps) parameter. Critical for successful video acquisition is the fact that the shutter speed of the camera should ideally be double the frames per second parameter. For instance, if recording is performed at 30 fps, the camera shutter speed should be locked at a value of approximately 60 (i.e., one-sixtieth of a second; Figure 1). When analyzing fast-moving objects and to avoid the heavy motion blur, the correct procedure is to increase the frame rate and in unison apply the correct shutter speed that is applicable for that frame rate. This is demonstrated in the detailed analysis of larval zebrafish twitching (Ono et al., 2002). Changing the shutter speed without properly adjusting the frame rate will result in issues such as choppiness of the video file and the risk of introducing banding and flickering on video files recorded under artificial illumination. Those effects can be particularly heavy when fluorescent lamps or light-emitting diode (LED) lights with low-frequency pulse width modulation controllers are used to adjust the intensity of light.

Lastly in specific circumstances both frames per second and shutter speed parameters might also need to be empirically optimized. This is particularly pertinent when using illumination sources that appear to "flicker" on the camera screen. "Flickering" occurs when the recording frame rate is higher than the cycle of electricity through the lighting circuit. The grid electricity operates on alternating current with a particular frequency such as 50 Hz (60 Hz in the United States), which means the circuit is turning on/off 100 (120 in the United States) times per second. Although not visible to the naked eye, that flicker can be seen through a camera lens when the shutter settings are not in sync with the hertz value of the main electricity that powers the illumination sources. To avoid such flicker, one can reduce the recording frame rate and adjust the shutter speed to closely match it to the hertz frequency (for 50- and 60-Hz grids a shutter speed divisible by 50 and 60, respectively). Some power supply drivers specifically designed for videography applications rectify the main current from 50 or 60 to 120 Hz, thus completely eliminating the issue. In general, battery-operated or direct-current lights are not plagued by such problems. However, as mentioned previously, the implementation of a pulse width modulation, or dimmer, which operates at a low frequency in the direct-current lighting circuit, can induce identical effects. Further information for implementing a pulse width modulation to optimize the background lighting is provided in the next sections.

Video file compression

Because the sequences of raw high-resolution videos consume large quantities of digital storage, data are often compressed using a range of available compression and decompression algorithms. The latter are commonly referred to as *codecs*, for example, H.264, MPEG, and ProRes. Based on selecting different camera settings, the compression can be performed to different standards that include selection of codecs as well as file digital containers. Two of the most used, universal, and efficient codecs are at present H.264 (for HD and UHD files) and H.265 (for UHD files). Importantly, video files recorded with the H.264 codec are accepted by the majority of commercial and open-source animal tracking software. As such, our recommendation is to predominantly set the recording cameras to use the H.264 codecs and avoid others that might require time-consuming digital transcoding for the files to be used in animal tracking software. The impacts of altering characteristics of video file compression in conjunction with animal tracking were discussed recently by Henry et al. (2019) and Henry and Wlodkowic (2020). Not all video containers are compatible with tracking suites; however, such videos are easily transcoded to enable tracking (Panadeiro et al., 2021). The pitfalls and process to transcode videos are noted below (Henry et al., 2019).

Some behavioral experiments require recording of video of very long duration. As a result, the generated video file sizes, despite using efficient codecs such as the previously mentioned H.264, will be considerable. Recording of long experiments on internal camera secure digital cards is suboptimal for many reasons including potential camera overheating, limitations in recording duration, as well as lack of professional scientific data archiving (Xia et al., 2018). As a result, we recommend using specialist external hardware to record video streams instead of capturing them on an internal camera storage card.

External recording hardware that uses HD multimedia interface (HDMI) is compatible with the majority of professional and even inexpensive prosumer cameras. In this regard, high-quality, robust, and user-friendly mirrorless interchangeable-lens cameras that support video recording of up to 4 K UHD resolution and can output clean HDMI stream can be nowadays obtained from diverse companies (e.g., Panasonic, Olympus, Sony, Canon, Nikon) at a very reasonable cost (often well below US$1000). Outputting HDMI signal by the camera bypasses its internal recoding circuits and allows recording of very long file durations (i.e., >24 h).

Our recommendations regarding the recording of HDMI video stream, if available funding allows, include dedicated and professional video production hardware such as the Atomos Ninja Inferno or the Convergent Design Odyssey 7Q+. These options, in our experience, are very good for filming experiments that last for many hours. They allow the users to record to fast solid state drive storage and are equipped with programmable features such as time-resolved filming (e.g., recording sequences of 15-min footage starting every hour). We recommend using HDMI cameras and matched HDMI recording hardware which require simple assembly and a highly efficient and robust video recording system. Those plug-and-play solutions provide a very flat learning curve, with practically no troubleshooting required and no engineering know-how.

When cost-saving considerations are critical, the HDMI signal from the camera can be also captured via inexpensive personal computer (PC) video capture cards, such as a family of Blackmagic HDMI Decklink Recorders, which enable video streams to be captured directly onto a desktop computer. In this scenario the PC-based recording can be completed using many freely available software, such as Open Broadcaster Software Studio (OBS Studio). In this scenario we recommend using PCs equipped with fast solid-state drives, quad-core or better processors, and random access memory of at least 16 gigabytes to achieve optimal data acquisition when recording HD and UHD videos.

In some instances, even cheaper solutions such as popular GoPro cameras and even Universal Serial Bus (USB)-web cameras can sometimes be viable options for recording animal behaviors. However, such cameras do not have interchangeable lenses and therefore lack the ability to attach macro and other lenses. Users should also be aware of their limitations because their sensors are usually subpar compared to even most inexpensive HDMI-equipped mirrorless interchangeable-lens cameras available on the market today with regard to light sensitivity, digital noise, sensor size, video frame rate, and bit rate recording. They can also be prone to overheating issues in long-term experiments.

It should be also mentioned that modern industry USB cameras can be very good options in particular for engineering-minded and/or multidisciplinary laboratories that are well versed in prototyping, programming, and software control layers. However, it has to be stated that such cameras are often significantly more expensive than most modern prosumer mirrorless interchangeable-lens cameras and often require specialist lenses, and the video acquisition is in many cases not as straightforward as is the case for the plug-and-play video production HDMI standard.

SOFTWARE-BASED ANIMAL TRACKING

Following the recording of digital files and associated considerations, a dedicated software is used to track animal locomotory activities. This process involves deconvolution of movement trajectories in a grid of pixels on each of the individual video frames. It enables users to extract and translate pixel information to quantitative behavioral endpoints, such as distance moved, velocity, and time spent in certain chamber zones (Henry & Wlodkowic, 2020).

Computer-enabled animal tracking using most commercial and open-source software packages predominantly involves contrast-based detection and subsequent center of mass (centroid) tracking of movement (Henry & Wlodkowic, 2020). This process requires a pixel intensity and/or color saturation threshold difference between the animals and the background. In other words, to be successfully detected and tracked, the animals need to be clearly distinguishable from the chamber background (Henry & Wlodkowic, 2020; Panadeiro et al., 2021). As such, one needs to strive to acquire videos where dark animals are positioned against a contrasting light background or vice versa. Moreover, any floating particles, scratches, or visible contamination of the tank surface and even digital noise due to incorrect ISO settings/suboptimal illumination that are of similar pixel intensity to the animal will generate detection and tracking bias. In some circumstances, it might not even be possible to track with sufficient fidelity. This is why optimization of digital video file settings and proper preparation and illumination of the test chambers are paramount for successful behavioral analysis.

INCREASING DATA FIDELITY THROUGH RIGOROUS STANDARDIZATION OF DIGITAL VIDEO ACQUISITION

The quality of the tracking data to be obtained is determined by the amount of effective pixel information assigned to each organism that can be extracted from the acquired video. As discussed in the preceding section, the majority of automated tracking software use contrast-based detection and center of mass tracking of movement. Therefore, the main priority in video recording of experiments is to ensure that the organism is uniformly illuminated and clearly contrasted against the chamber background at all times (Figure 2). The following guidelines are key to increasing the quality of the behavioral data that can be generated using tracking software.

Organism size considerations

The advent of animal tracking software capabilities, such as multiarena analysis, provides opportunities to develop very powerful analytical capabilities for high-throughput behavioral biotests, especially in rapid chemical prioritization for further testing (Bownik & Wlodkowic, 2021; Henry & Wlodkowic, 2019, 2020). Multiarena tracking denotes the ability of the software to simultaneously and independently track animals kept in different test chambers that have been recorded on one video file. A very good example of this powerful analytical capability is analysis of small aquatic animals kept in multiwell plates, multiple Petri dishes, or small tanks. At present, software solutions already exist that enable all animals to be tracked simultaneously, even in 96 independent test wells. This provides very powerful capability to simultaneously analyze, for instance, multiple independent replicate treatments across many samples. Such high-throughput biotests commonly utilize small aquatic model organisms that are widely accepted in ecotoxicology (e.g., *Daphna magna*, *Artemia franciscana*, *Gammarus* sp., larval stages of *Danio rerio*) that can be kept in multiwell laboratory test plates. The size of such organisms provides, however, some unique challenges for behavioral biotests in terms of quality and resolution of video recording; namely, imaging of the above-mentioned 96 independent test wells with organisms <1 mm will require applications of HD or UHD (4 K) camera resolutions when all test wells are to be imaged simultaneously. This is because the majority of commonly used animal tracking software requires an object to be approximately 50 px in size to be detected in a standard video frame. It logically follows that as more objects are placed in the

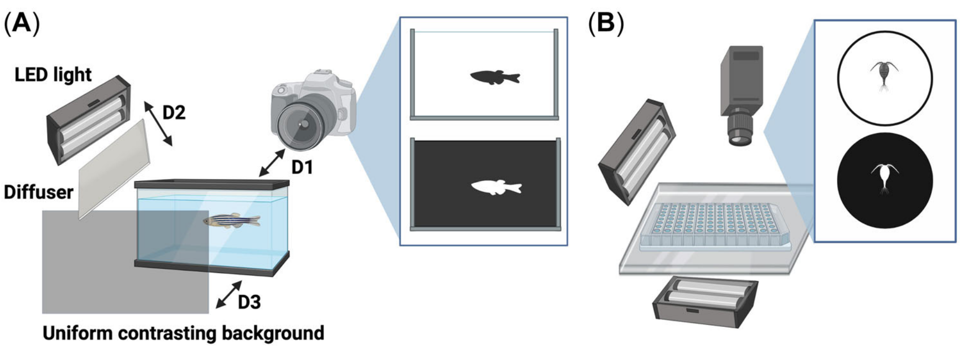

FIGURE 2: Illumination and chamber placement. The main priority in video recording of most behavioral experiments is to ensure that the organisms are uniformly illuminated and clearly always contrasted against the chamber background. Best for software animal tracking are videos where dark animals are positioned against a contrasting light background or vice versa irrespective of whether recording in (**A**) large tanks or (**B**) multiwell plates. Uniformly diffused, soft light is the gold standard for behavioral experiments. LED = light-emitting diode.

frame to be tracked, the overall resolution of the frame must be higher for each object's size to be above the 50-px minimum.

Video camera mounting and stability

Camera mounting above the target imaging area must be highly stable (Figure 1C). Some experiments, such as tank diving testing or when using multiple cameras to track three-dimensional movement of fish, also require cameras to be located in front of the targeted imaging area (Fontana et al., 2022; Francisco et al., 2020; Haghani et al., 2019). It is of key importance that the camera is fixed and cannot be shifted, even with minor vibrations, during recording, to prevent a plethora of tracking errors. Camera stability ensures that the video is correctly focused for the entire duration of the experiment. This is also imperative when running replicate experiments to be compared because the camera and organism chamber must be always fixed relative to each other for the calibration to real-world distance to remain constant (Figure 2). It is recommended to place markers at set distances within the filming arena to allow for calibration to real-world distances for consistency; the benefit of fixing the camera and chamber in place is a reduction in user time in recalibrating the tracking software prior to commencing tracking. Thus, fixing the camera and chamber position is key for high-throughput studies. If postprocessing is required for other reasons, as discussed in detail throughout, any shifts in camera position will contribute to substantial additional processing time and even potentially make the video unable to be tracked if the organism arena was to shift outside the set tracking area during the video. Depending on the lighting apparatus, the camera shifting position could also be interpreted as a stimulus to the target organism and add unintended variables to the results.

Video camera settings considerations

All video camera settings should be always locked for the entire duration of the experiment as well as for all experiments that represent the same set. In other words, the camera's aperture, shutter speed, ISO, and frame rate as well as codec selection should always be kept the same for all video files recorded if the animal tracking data are to be reliably compared. One should at all costs avoid common pitfalls such as placing the camera in automatic mode. The latter will continually adjust and change the camera settings during the recording, making consistent detection settings in later tracking difficult as the background pixel intensity shifts with the focus.

Camera resolution plays a key role especially in experiments where multiple chambers each holding multiple, especially small organisms are imaged simultaneously. In general, the higher the resolution of the sensor, the higher the number of pixels will be available for detecting the organism. Increasing the resolution can thus vastly improve the overall accuracy of the animal tracking. Previous work has described how increasing the pixel number increases the number of pixels allocated to the organism relative to the background (Henry & Wlodkowic, 2020). Most animal tracking software available today requires a minimum of 50 px assigned per organism for optimal tracking (Henry & Wlodkowic, 2020; Rodriguez et al., 2018). As a result, tracking multiple small organisms in multiple chambers usually calls for modern HD or even UHD cameras. These cameras are key to unlocking high-throughput behavioral ecotoxicity testing (Henry & Wlodkowic, 2020).

Maintaining the same frame rate through any processing or recording of trials is also of high importance. Although transcoding to a different frame rate is possible using video editing software, this should be avoided at all costs. Recent work has demonstrated that transcoding a video file filmed at 60 fps down to 30 fps led to loss of half of the endpoint data and inaccurate tracking (Henry et al., 2019). The awareness of the need to maintain frame rate can prevent significant analytical errors.

Camera lens considerations

A commonly ignored aspect is selection of the camera lens. In all experimental series the lens (or lens focal length for the popular zoom lenses) should be always kept the same. It is also paramount to disable any autofocus; always set the focus manually. This is because autofocus systems might introduce significant temporary shifts of focus, in turn producing random sequences of video that are out of focus, and thus animals unable to be tracked subsequently using dedicated software. Setting focus manually avoids these all-too-common issues. Another recommendation is to disable any optical image stabilization systems the camera might be equipped with such as optical lens or active sensor shift technologies. These technologies have been designed to stabilize the image when the camera is in motion. When the camera is, however, mounted stationary and immobile the image stabilization systems themselves can often become a source of "shaky videos" and undesired wandering of the video frames that will make computer-based tracking of animals very difficult, if not impossible. This issue is particularly noticeable with cameras employing active sensor shift technology, and per manufacturer recommendations, such systems should be disabled when the camera is stationary, to avoid such artifacts. Moreover, where possible the distance between the front lens element and the test chamber should also be kept constant between all experiments that are to be compared. As previously noted, this will reduce time in calibrating the tracking software in large-scale behavioral experiments including applications of batch processing of video files and multiarena animal tracking.

It is important to note that wide focal length lenses are prone to introducing parallax errors, which cause the apparent position of the object to be displaced in the recording as a result of the lens viewing angle not being perpendicular to the object across all areas in a given arena. These strong image distortions can even lead to the object becoming hidden from view. This can be avoided by implementation of special telecentric lenses that will help reduce this perspective distortion.

Alternatively, using a greater focal length and placing the camera farther away from the test chamber will help in alleviating optical distortions to some degree.

Test chamber preparation

In each experiment to be recorded it is imperative to include a visible indicator in the field of view that has a known and quantifiable length. This is best included at the periphery of the imaged arena. This will allow for the calibration of pixel data to real-world distances for tracking data analysis. Some tracking suites allow for automating this calibration process (Rodriguez et al., 2018). However, using optical gridlines on the background designed for manual scoring should be avoided when utilizing automated tracking software. The dark lines of such grid patterns consistently fall within the organism pixel detection threshold and introduce a plethora of detection errors because the software switches the detected center of mass across the gridline sections of the entire chamber (Beran et al., 2016). This makes the tracked organism largely indistinguishable from the background (Figure 3). These gridlines can be removed with special background subtraction postprocessing techniques; however, this is not a preferable solution (Beran et al., 2016).

The position between the test chamber and the camera should also be kept constant. Even any minor shifts or disruptions to the camera and/or chamber position during recording or between different experiments will require additional calibration work to make subsequent replicate recordings usable for a comparative analysis.

When conducting experiments on organisms that are significantly smaller than the chamber volume (e.g., small invertebrates, larval stages of fish or amphibians), the meniscus of the medium in the test chamber should be kept slightly convex. This will greatly minimize or even eliminate the occurrence of shadows at the circumference of the chamber. In those regions the animals can hide from the view of the camera and become undetectable for animal tracking software because of lack of sufficient contrast values required for their accurate detection. Special consideration to this problem should be given when conducting long-duration trials because evaporation from the chambers will induce a concave meniscus and thus introduce periphery/edge shadows. The evaporation can be minimized by humidifying the environment or adding liquid in between experiments. Alternatively, the implementation of microperfusion systems designed for behavioral experiments such as those presented recently by Bai et al. (2020) and Huang et al. (2016) can help to eliminate the evaporation artifacts.

The test chambers should preferably be of good condition without any imperfections such as visible scratches and dirt particles that can interfere with animal detection and tracking (Figure 3). The test chamber material should be homogenous,

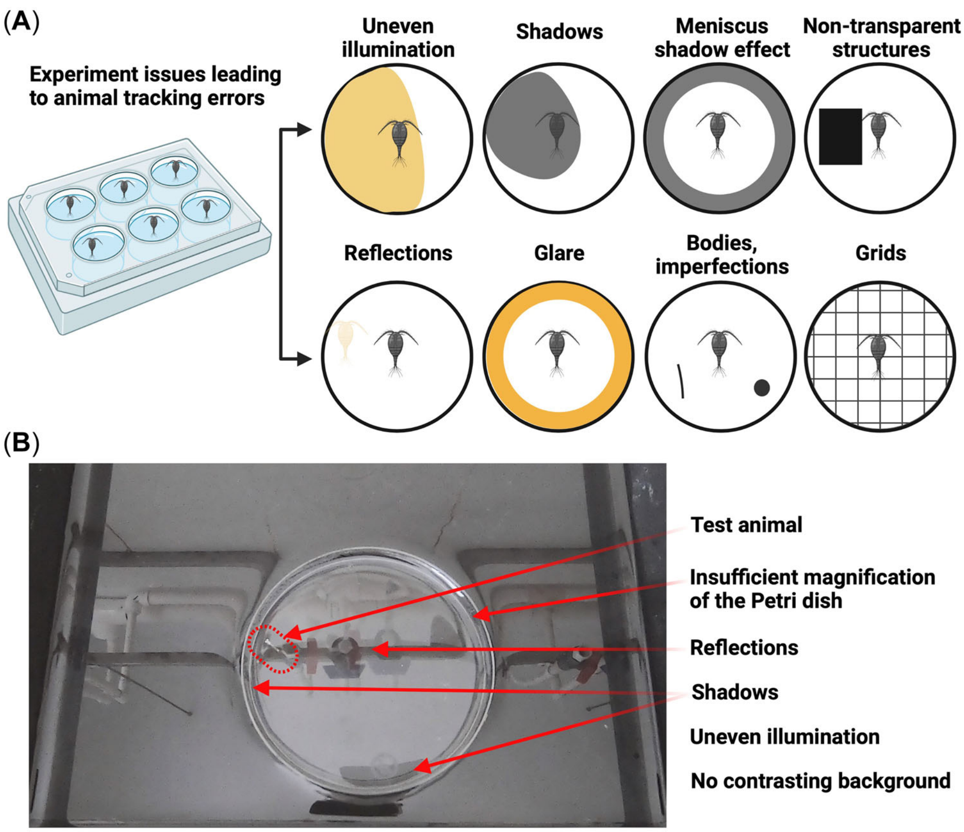

FIGURE 3: Common pitfalls in setup of behavioral experiments for software-based animal tracking. (**A**) Potential video footage issues that can lead to significant animal tracking errors and data loss. (**B**) An example of suboptimal footage with insufficient magnification, excessive reflections, shadows, and uneven illumination.

and where possible no sharp edges with alternating materials should be used.

Illumination of test chambers

Any artifacts in the background illumination that fall into animal pixel intensity threshold range might cause the software to track the artifact rather than the target organism. All video recording for behavioral analysis must thus ensure as consistent and uniform illumination of the test chambers as possible. Uniformly diffused, soft light is the gold standard for behavioral experiments, irrespective of whether illumination is performed by white or IR light sources. Light diffusers can be acquired from diverse sources, and they should be empirically tested to assure optimal dispersal of light.

The light source must also be constant in its intensity and color (wavelength), and it must be identically placed between all experiments. When using large fish tanks, it is highly advisable to mount lights on professional studio grade-C stands or on custom overhead rails. Marking placement of light on the floor is also a good practice, to ensure consistency between experiments. If additional illumination intensity is required to achieve consistent exposure, the lighting of the chamber should be increased rather than compensating through altering camera settings such as ISO. Altering ISO/gain levels can substantially increase pixel noise, which can also be construed as artifacts and lead to incorrect assignment in animal detection.

Special care should be taken to avoid any glares, ripples, and reflections on the medium surface of the aquatic test chamber (Figure 3). These dynamic ripples and reflections have a significant potential to fall within the organism pixel detection parameters when tracking. In this case tracking suites with adaptive thresholding capability can be utilized, or the entire video must be viewed in tracking to ensure that organism tracking identity is maintained. Video files with suboptimal illumination or illumination artifacts still have the potential to be tracked; however, they will require substantial video postprocessing and noise reduction techniques applied in dedicated video editing software (see below). This additional time investment can be easily avoided by thorough optimization of the initial recording setup.

Lastly, the illumination sources should not introduce any temperature changes in the test chambers. In general, based on our experience, we recommend LED illumination sources that can be controlled by high frequency pulse width modulation controllers. As discussed previously, high frequency pulse width modulation controllers are required to ensure that the cycle of electricity through the lighting circuit is higher than the recording frame rate to prevent the "flickering" effect. This is of particular importance when recording at high frames per second and shutter speeds. Both LEDs and pulse width modulation controllers are these days very inexpensive and can be purchased online from many vendors. We particularly recommend flexible LED strips as a convenient solution that offers freedom in constructing custom illumination sources at very affordable cost.

UTILIZING DIGITAL VIDEO POSTPROCESSING TECHNIQUES TO IMPROVE TRACKING CONSISTENCY

Postprocessing of videos typically involves altering (editing) specific aspects of the native recording using the dedicated video editing software. We recommend the cross-platform and freely available DaVinci Resolve 16 (BlackMagic Design) software for any postprocessing tasks. There are, of course, many alternatives for all major operating systems including even applications on mobile devices.

As a general rule for all behavioral assays, the time length of the recording should be edited so that all replicate videos are of equal duration. This also means ensuring that any stimulus interactions taking place in the assay are conducted at the same time point (Kokel et al., 2010; Schnörr et al., 2012).

Both the frame rate and resolution settings of the transcoded video should be maintained consistent with raw videos from the camera, as discussed previously. The transcoding can be, however, implemented to generate video files encoded using specific codecs and enclosed in video containers that are acceptable inputs for animal tracking software. It should be noted that this is often required when using some commercial external HDMI recording hardware that does not feature built-in video encoders. They thus generate uncompressed files (e.g., ProRes standard) of very large size that must be then compressed during the digital postprocessing stage. Generally, video files compressed using popular H.264 codec and saved in .mp4 containers (formats) are the most universal medium for most animal tracking software currently available.

As discussed, tracking errors are often introduced through uneven illumination, shadows, and reflections during behavioral experiments. This can introduce significant obstacles for reproducible video-based animal tracking if such artifacts are the same pixel intensity and size of the target organism in the recordings. In some circumstances, it might not even be possible to analyze the suboptimally recorded video files using computer-based animal tracking. If this has occurred and the artifacts are stationary, they can in many instances be removed using digital background subtraction techniques. We have developed a very straightforward workflow that can be applied in most video editing software for this purpose. It eliminates the native background and replaces it with a clean white (positive subtraction) or black (negative subtraction) background with corresponding sharply contrasting animals (Figure 4). Utilizing the freely available DaVinci Resolve 16, the digital workflow involves creating a mask file, which is a still image from a frame of the video with lines drawn marking the calibration distance and a completely white area denoting the target tracking arena. The next step is to convert another single frame, usually a last second of the video (a time point in the video that will not be tracked), into a still frame. In the video editing software this frame is layered above and subtracted from every frame in the original video (Figure 4). The mask is then layered above this and differentiated from every frame in the video. This creates an arena that has a completely white background area where the organism will be dark

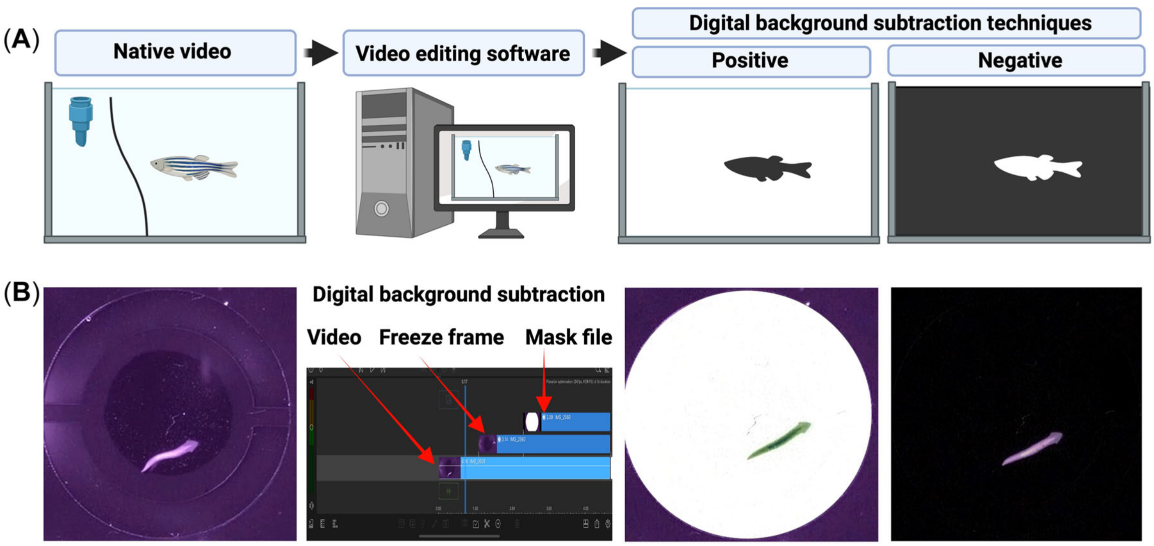

FIGURE 4: Digital background subtraction technique aimed at enhancing the footage for high-fidelity animal tracking. (**A**) Cartoon depicting workflow of the technique that utilizes video editing software. (**B**) An example of experimental footage processed to obtain positive and negative background subtraction. The digital workflow involves creating a mask file, which is a still image from a frame of the video with white or black area denoting the target tracking arena. The next step is conversion of a last-second frame of the video (a time point in the video that will not be tracked) into a still frame. In the video editing software this frame is layered above and subtracted from every frame in the original video. The mask is then layered above this and differentiated from every frame in the video.

(Figure 4). The calibration lines are important to enable the tracking results to be properly calibrated in the tracking software. We have demonstrated that the digital background subtraction techniques can increase consistency of animal tracking results as well as rescue video files that were previously unusable for tracking (Henry et al., 2019).

Moreover, if video artifacts have been caused by high ISO/gain settings, appearing very grainy or flickering, one can utilize DaVinci Resolve 16 to employ advanced temporal and spatial noise reduction filters in the postprocessing stages (Figure 5A,B; Supporting Information, Videos S1 and S2). Temporal noise reduction compares consecutive frames to identify the noise

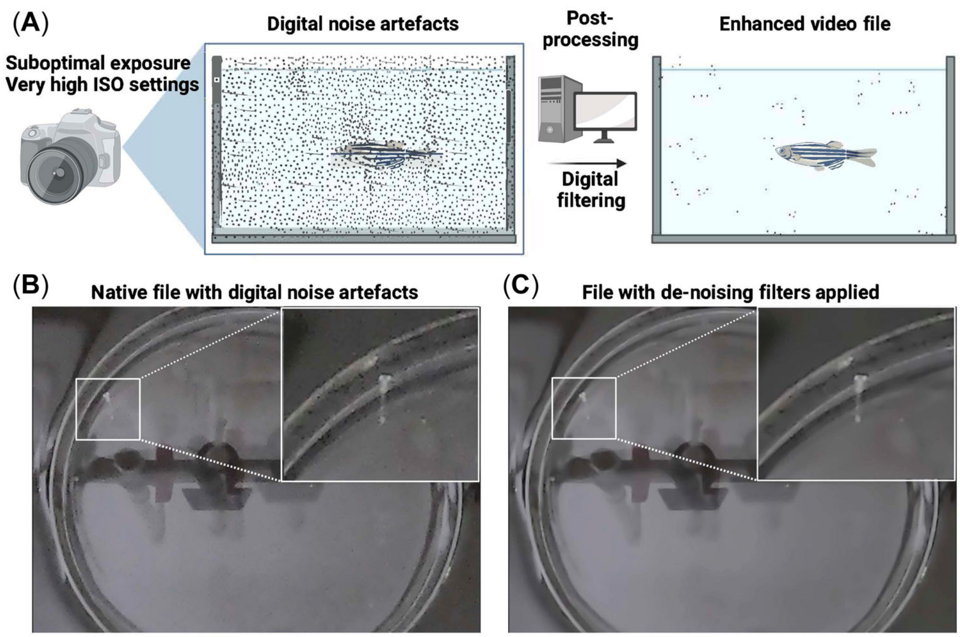

FIGURE 5: Digital noise reduction techniques aimed at optimization of very grainy or flickering video footage caused by high ISO/gain settings, insufficient intensity, or flickering of illumination. (**A**) Cartoon depicting workflow of the technique that utilizes video editing software with a specialist plug-in for noise reduction. (**B**) An example of experimental footage with high levels of digital noise (see also Supporting Information, Video S1). (**C**) The experimental footage processed with de-noise filters in the Final Cut Pro X software equipped with a NeatVideo plug-in (see also Supporting Information, Video S2). ISO = International Organization for Standardization.

level according to frame-to-frame intensity differences and applies a filter on all frames. Spatial noise techniques create a noise profile according to a designated reference region on one frame and then smooth out the reset regions based on the noise profile. The spatial filter can be stored and applied in filtering of subsequent frames. Such filters can be used to reduce and smooth pixel intensity fluctuations associated with noisy videos (Supporting Information, Video S2). Such processing often requires specialized software plug-ins but offers very powerful capability to eliminate most digital noise, flicker, and other video imperfections (very good, low-cost options are OpenFX Reduce Noise v5 and Neat Video; Figure 5B; Supporting Information, Video S2). Further contrast adjustment and background subtraction techniques can also be applied afterward if required.

CONCLUSIONS

In the present study our aim was to draw attention to the important topic of video data acquisition and illustrate key concepts in video-based behavioral analysis. The present study was spurred by the fact that the actual importance of video quality in obtaining high-fidelity and reproducible behavioral data sets is still greatly underappreciated in the field. Proper setup for successful behavioral experiments is usually a little bit more complex than most researchers initially anticipate. It involves selecting and locking the optimal in-camera settings as well as assuring highly consistent and reproducible camera mounting, uniformly soft illumination, and test chamber clarity during all experiments. The old analog photographer's mantra of the 20th century "get it right in the camera" holds very true and will assure only limited need for video postprocessing steps.

It is our hope that the above guidelines and techniques will provide a solid foundation for many scientists interested in commencing and/or improving their behavioral experimental workflows. Understanding the basic concepts of digital video acquisition and adhering to some fundamental videography rules will save a lot of time and effort while preventing significant errors and disappointments at the stage of computer-based animal tracking.

We welcome all collaborative enquiries and are happy to provide advice and share our ever-improving digital workflows and optimization techniques to enable wider standardization and better optimization of video data in behavioral aquatic ecotoxicology.

Supporting Information—The Supporting information are available on the Wiley Online Library at https://doi.org/10.1002/etc.5434.

Acknowledgment—Part of the present study was presented during the SETAC Australasia 2021 conference online and received the Best Student Poster Award. Figures were created with BioRender.com. Open access publishing facilitated by RMIT University, as part of the Wiley - RMIT University agreement via the Council of Australian University Librarians.

Author Contributions—**Jason Henry:** Formal analysis, Methodology, Software, Writing original draft. **Yutao Bai:** Formal analysis, Methodology, Software Donald Wlodkowic: Conceptualization, Methodology, Project Administration, Resources, Supervision, Visualization, Writing–review & Editing.

Conflict of Interest—The authors declare no conflict of interest.

Data Availability Statement—There are no experimental data.

REFERENCES

Ågerstrand, M., Arnold, K., Balshine, S., Brodin, T., Brooks, B. W., Maack, G., McCallum, E. S., Pyle, G., Saaristo, M., & Ford, A. T. (2020). Emerging investigator series: Use of behavioural endpoints in the regulation of chemicals. *Environmental Science and Processes Impacts*, *22*(1), 49–65. https://doi.org/10.1039/C9EM00463G

Bai, Y., Henry, J., Campana, O., & Wlodkowic, D. (2021). Emerging prospects of integrated bioanalytical systems in neuro-behavioral toxicology. *Science of the Total Environment*, *756*, Article 143922. https://doi.org/10.1016/j.scitotenv.2020.143922

Bai, Y., Henry, J., & Wlodkowic, D. (2020). Chemosensory avoidance behaviors of marine amphipods *Allorchestes compressa* revealed using a millifluidic perfusion technology. *Biomicrofluidics*, *14*(1), Article 014110. https://doi.org/10.1063/1.5131187

Beran, L., Chmelar, P., & Rejfek, L. (2016). Image processing methods usable for object detection on the chessboard. *MATEC Web of Conferences*, *75*(3), Article 03004. https://doi.org/10.1051/matecconf/20167503004

Bownik, A., & Wlodkowic, D. (2021). Applications of advanced neurobehavioral analysis strategies in aquatic ecotoxicology. *Science of the Total Environment*, *772*, Article 145577. https://doi.org/10.1016/j.scitotenv.2021.145577

Brooks, J. S. (2009). The emergence of behavioral testing of fishes to measure toxicological effects. *Toxicology Research*, *25*(1), 9–15. https://doi.org/10.5487/TR.2009.25.1.009

Final Cut Pro X [Computer software]. Version 10.6.3. Apple.

Fontana, B. D., Alnassar, N., & Parker, M. O. (2022). The zebrafish (*Danio rerio*) anxiety test battery: Comparison of behavioral responses in the novel tank diving and light–dark tasks following exposure to anxiogenic and anxiolytic compounds. *Psychopharmacology*, *239*(1), 287–296. https://doi.org/10.1007/s00213-021-05990-w

Ford A. T., Ågerstrand M., Brooks B. W., Allen J., Bertram M. G., Brodin T., Dang Z., Duquesne S., Sahm R., Hoffmann F., Hollert H., Jacob S., Klüver N., Lazorchak J. M., Ledesma M., Melvin S. D., Mohr S., Padilla S., Pyle G. G., … Maack G. (2021). The role of behavioral ecotoxicology in environmental protection. *Environmental Science & Technology* *55*(9):5620–5628. https://doi.org/10.1021/acs.est.0c06493

Francisco, F. A., Nührenberg, P., & Jordan, A. (2020). High-resolution, noninvasive animal tracking and reconstruction of local environment in aquatic ecosystems. *Movement Ecology*, *8*(1), Article 27. https://doi.org/10.1186/s40462-020-00214-w

Gaw, S., Thomas, K. V., & Hutchinson, T. H. (2014). Sources, impacts and trends of pharmaceuticals in the marine and coastal environment. *Philosophical Transactions of the Royal Society of London Series B Biological Sciences*, *369*(1656), Article 20130572. https://doi.org/10.1098/rstb.2013.0572

Grandjean, P., & Landrigan, P. (2006). Developmental neurotoxicity of industrial chemicals. *Lancet*, *368*, 2167–2178.

Haghani, S., Karia, M., Cheng, R.-K., & Mathuru, A. S. (2019). An automated assay system to study novel tank induced anxiety. *Frontiers in Behavioral Neuroscience*, *13*, Article 180. https://doi.org/10.3389/fnbeh.2019.00180

Henry, J., Bai, Y., Kreuder, F., Mawdsley, D., Kaslin, J., & Wlodkowic, D. (2022). Accelerating chemobehavioral phenotypic screening in neurotoxicology using a living embryo array system. *Zebrafish*, *19*(1), 32–35. https://doi.org/10.1089/zeb.2021.0072

Henry, J., Rodriguez, A., & Wlodkowic, D. (2019). Impact of digital video analytics on accuracy of chemobehavioural phenotyping in aquatic toxicology. *PeerJ*, *7*, Article e7367. https://doi.org/10.7717/peerj.7367

Henry, J., & Wlodkowic, D. (2019). Towards high-throughput chemobehavioural phenomics in neuropsychiatric drug discovery. *Marine Drugs*, *17*(6), Article 340. https://doi.org/10.3390/md17060340

Henry, J., & Wlodkowic, D. (2020). High-throughput animal tracking in chemobehavioral phenotyping: Current limitations and future perspectives. *Behavioural Processes*, *180*, Article 104226. https://doi.org/10.1016/j.beproc.2020.104226

Huang, Y., Persoone, G., Nugegoda, D., & Wlodkowic, D. (2016). Enabling sub-lethal behavioral ecotoxicity biotests using microfluidic Lab-on-a-Chip technology. *Sensors and Actuators B: Chemical*, *226*, 289–298. https://doi.org/10.1016/j.snb.2015.11.128

Kalueff, A. V., Echevarria, D. J., Homechaudhuri, S., Stewart, A. M., Collier, A. D., Kaluyeva, A. A., Li, S., Liu, Y., Chen, P., Wang, J., Yang, L., Mitra, A., Pal, S., Chaudhuri, A., Roy, A., Biswas, M., Roy, D., Podder, A., Poudel, M. K., … International Zebrafish Neuroscience Research Consortium. (2016). Zebrafish neurobehavioral phenomics for aquatic neuropharmacology and toxicology research. *Aquatic Toxicology*, *170*, 297–309. https://doi.org/10.1016/j.aquatox.2015.08.007

Kohler, S. A., Parker, M. O., & Ford, A. T. (2018). Shape and size of the arenas affect amphipod behaviours: Implications for ecotoxicology. *PeerJ*, *6*, Article e5271. https://doi.org/10.7717/peerj.5271

Kokel, D., Bryan, J., Laggner, C., White, R., Cheung, C. Y., Mateus, R., Healey, D., Kim, S., Werdich, A. A., Haggarty, S. J., Macrae, C. A., Shoichet, B., & Peterson, R. T. (2010). Rapid behavior-based identification of neuroactive small molecules in the zebrafish. *Nature Chemical Biology*, *6*(3), 231–237. https://doi.org/10.1038/nchembio.307

Legradi, J. B., Di Paolo, C., Kraak, M. H. S., van der Geest, H. G., Schymanski, E. L., Williams, A. J., Dingemans, M. M. L., Massei, R., Brack, W., Cousin, X., Begout, M. L., van der Oost, R., Carion, A., Suarez-Ulloa, V., Silvestre, F., Escher, B. I., Engwall, M., Nilén, G., Keiter, S. H., … Hollert, H. (2018). An ecotoxicological view on neurotoxicity assessment. *Environmental Sciences Europe*, *30*(1), Article 46. https://doi.org/10.1186/s12302-018-0173-x

Melvin, S. D., & Wilson, S. P. (2013). The utility of behavioral studies for aquatic toxicology testing: A meta-analysis. *Chemosphere*, *93*(10), 2217–2223. https://doi.org/10.1016/j.chemosphere.2013.07.036

Ono, F., Shcherbatko, A., Higashijima, S., Mandel, G., & Brehm, P. (2002). The zebrafish motility mutant *twitch once* reveals new roles for rapsyn in synaptic function. *Journal of Neuroscience*, *22*(15), 6491–6498. https://doi.org/10.1523/JNEUROSCI.22-15-06491.2002

Panadeiro, V., Rodriguez, A., Henry, J., Wlodkowic, D., & Andersson, M. (2021). A review of 28 free animal tracking software: Current features and limitations. *Lab Animal*, *50*(9), 246–254.

Paskin, T. R., Jellies, J., Bacher, J., & Beane, W. S. (2014). Planarian phototactic assay reveals differential behavioral responses based on wavelength. *PLOS ONE*, *9*(12), Article e114708. https://doi.org/10.1371/journal.pone.0114708

Rodriguez, A., Zhang, H., Klaminder, J., Brodin, T., Andersson, P. L., & Andersson, M. (2018). ToxTrac: A fast and robust software for tracking organisms. *Methods in Ecology and Evolution*, *9*(3), 460–464. https://doi.org/10.1111/2041-210X.12874

Schnörr, S. J., Steenbergen, P. J., Richardson, M. K., & Champagne, D. L. (2012). Measuring thigmotaxis in larval zebrafish. *Behavioural Brain Research*, *228*(2), 367–374. https://doi.org/10.1016/j.bbr.2011.12.016

Walpitagama, M., Carve, M., Douek, A. M., Trestrail, C., Bai, Y., Kaslin, J., & Wlodkowic, D. (2019). Additives migrating from 3D-printed plastic induce developmental toxicity and neurobehavioural alterations in early life zebrafish (*Danio rerio*). *Aquatic Toxicology*, *213*, Article 105227. https://doi.org/10.1016/j.aquatox.2019.105227

Xia, C., Fu, L., Liu, Z., Liu, H., Chen, L., & Liu, Y. (2018). Aquatic toxic analysis by monitoring fish behavior using computer vision: A recent progress. *Journal of Toxicology*, *2018*, Article 2591924. https://doi.org/10.1155/2018/2591924

Zala, S. M., & Penn, D. J. (2004). Abnormal behaviours induced by chemical pollution: A review of the evidence and new challenges. *Animal Behaviour*, *68*(4), 649–664. https://doi.org/10.1016/j.anbehav.2004.01.005

